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domains

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#### Introduction

Understanding the molecular and cellular mechanisms that trigger breast cancer is essential to the prevention and treatment of this disease. The BRCA1 C-terminal (BRCT) domain, was first identified in BRCA1 (1, 3). Cancer associated missense and deletion mutations have been found in the BRCT repeat regions of BRCA1, suggesting an important role of BRCT domains in regulating BRCA1 activity (16, 23). In addition, the BRCT domain is found in many proteins that regulate DNA damage repair, cell cycle, and genome stability, implying a more global role of BRCT domains in genome stability surveillance (1, 3). Consistent with this notion, the BRCT domain has been shown to mediate protein-protein interactions. For example, BRCT domains of BRCA1 associate with helicase BACH1 and CtBP interacting protein CTIP (4, 49). Recently, our lab and others have discovered that BRCT domains are novel phosphopeptide binding modules (20, 32, 48). BRCA1 BRCT domains associate with residue Ser990 on BACH1 in a phosphorylation-dependent manner. Furthermore, we found that several other BRCT domains including those from MDC1 and tumor suppressor BARD1 can bind specific phosphorylated peptides (12, 19, 38). These findings suggest that the BRCT domain recruits phosphorylated cellular targets and mediates signaling complex formation. However, the identities of the in vivo BRCT domain targets are largely unknown. In this application, we propose to use peptide libraries and peptide arrays to systematically identify phosphoproteins that can interact with BRCT domains. We may uncover potential new regulators of genome stability; more importantly, the approach can identify phosphorylated sequences on proteins that are important for DNA damage responses and cell cycle. Such information will help us to understand the mechanism of how protein phosphorylation modulates DNA damage responses and cell cycle in breast epithelial cells. In addition, it should prove invaluable for the development of new screening strategies and treatment for breast cancer.

# Body

- A. For Task 1, we proposed to identify phosphorylated peptide sequences that specifically bind BRCA1 and BARD1 BRCT domains. This will be achieved by (1) establishing Oriented Peptide Array Libraries (OPAL) and using OPAL to examine the specificity of BRCA1 and BARD1, and (2) by establishing peptide chips to screen for BRCA1 and BARD1 BRCT domain binding targets.
- 1) For the first part of Task 1, we focused on BRCT domain fusion proteins and OPAL arrays, both of which are key reagents.
- 1.1 First generation OPAL was syntheized on cellulose paper and worked well with SH2 domains and kinases. We therefore carried out analysis of BRCA1 and BARD1 BRCT domain fusion proteins using cellulose paper based OPAL arrays. The set up of OPAL is depicted in Figure 1a (left panel) and the results of BARD1 BRCT domain is shown in Figure 1 (right panel). Clearly, the BARD1 BRCT domain had no specific sequence preference for the amino terminus of the peptide. Furthermore, it showed preference for Asp and Glu residues at the carboxy terminus. This result is conssistent with our published finding that BARD1 prefers acidic residues at position C-terminus to the phosphoserine

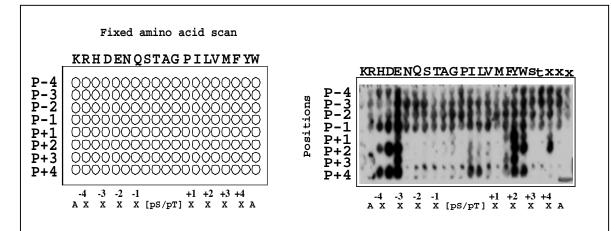


Figure 1. Left, OPAL strategy. A library AXXXX[pS/pT]XXXXA is synthesized on cellulose membranes. The X position is substituted by one of the 19 amino acids (except Cys). A total 152 library pools are synthesized for the array showing. Right, OPAL analysis of BARD1 BRCT domain.

using soluble oriented peptide libraries (9). However, these arrays generally had very high background. For instance, nono-specific binding to F, W, and H. Furthermore, many of the "interactions" observed appeared to arise between the cellulose surface and BRCT fusion proteins, rather than between the spotted phopshopeotides and BRCT fusion proteins.

To solve the non-specific binding and high background problem, we are establishing different approaches to synthesize OPAL. One method is to increase the distance bewteen phsophoserine and the cellulose surface. This can be achieved by adding longer linkers on the cellulose surface. Another method is to synthesize soluble peptide libary pools and then spot the peptide libraries on different solid supports such as glass. These changes represent significant changes to our established protocols. However, we do not forsee major setbacks because numerous studies have been done to optimize peptide synthesis on different surfaces such as glass.

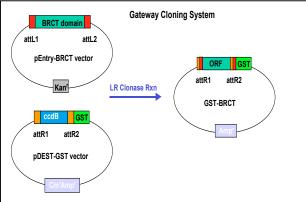


Figure 2. Schematic representation of the Gateway cloning system.

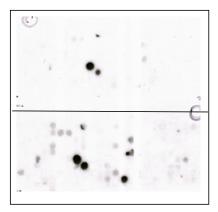
1.2 We are in the process of cloning and constructing expression vectors for all the BRCT domains in the human genome. To accomplish this, we are utilizing the Gateway® cloning system ((Invitrogen). In this case, we have collected several BRCT domain cDNAs in Gateway pENTR vectors, and engineered a collection of epitope-tag destination vectors (e.g., GST) (Figure 2). The various BRCT domains can then be conviniently shuffled into the destination vectors via clonase-mediated recombination.

2) The second part of Task 1 seeks to screen for BARD1 binding peptides. To accomplish this, we systhesized an array (peptide chip) containing ~500 potential DNA damage phosphorylation sites selected from a number of proteins (Figure 3). We performed binding assays using BARD1BRCT domain and control proteins. The approach was validated by our identification of a number of target sites. For example, phopshopeptide sequences from BACH1 were identified for BRCA1 BRCT domain using this method.

### Criteria for selecting phosphorylation sequences:

- 1. Proteins are involved in DNA damage response.
- 2. Sequences show high conservationthroughout evolution (e.g., from fish to mice and human).
- 3. The sequences agree with known kinase target sites.

Sample list of Proteins from which sequences were selected: BRCA1, Chk, Chk2, BACh1, MDC1



**BARD1 BRCT domain** 

Non-BRCT domain ctr

32x16 peptide chip

Figure 3. Top, selection criteria for the peptide sequences selected. Bottom, two peptide chips were probed with BRAD1 BRCT domain or a control domain.

There were a few reactive spots on the array when probed with BARD1 (Figure However, the same spots were also positive in the control array experiments probed with none-BRCT proteins. Therefore. οf these none phosphopeptides bound specifically to BARD1 BRCT domain, suggesting that they are not targets of BARD1. We are currently generating larger arrays or developing new methods to identify BARD1 BRCT targets.

B. For Task 2, we proposed to characterize breast cancer genes and

BRCT binding sites identified in Task 1, by RNAi and by determining the role of BRCT-phosphopeptide interaction in DNA damage. While we are optimizing screening conditions and generating assay reagents, we have conducted more detailed analysis of BARD1.

Three retroviral RNAi vectors for BARD1 have been made. We also have obtained two different antibodies for human BARD1. We currently analyzing these antibodies to make sure that they can both immunoprecipitate and western blot BARD1, which should help us to identify the RNAi vectors that significantly knockdown BARD1 expression.

### **Key Research Accomplishments**

A number of key findings have resulted from the work so far:

- Analyzed BRCA1 and BARD1 BRCT domains using OPAL spotted on cellulose
- Analyzed BARD1 BRCT domain using peptide chips containing ~500 peptides
- Constructed RNAi vectors for BARD1
- Engineered Gateway pENTR and Destination vectors for epitope-tagged BRCT domain expression
- Prelininary data on BARD1 BRCT binding sites
- Demonstrated feasibility of using OPAL to screen for phospho-binding sites
- · Identification of problems associated with cellulose based binding assays
- We have already obtained and verified a collection of GST-BRCT domain fusion proteins
- We showed feasibility of peptide chips containing ~500 peptides for binding analysis

### **Reportable Outcomes**

We have utilized the OPAL and peptide chips approach to investigate the binding specificities of BRCA1 and BARD1 BRCT domains. A motif for BARD1 BRCT domain recognition was identified.

# Meeting abstract/presentation

Specificity of BRCT domains and breast cancer. (2005) Maria Rodriguez, Zhou Songyang. SCBA Annual Research Conference. Houston, TX

#### Conclusions

In summary, we have conducted pilot screens of BRCT domain interacting sequences using peptide chips. Furthermore, we are in the process of optimizing OPAL for BRCT interaction screens. We determined that a different surface (other than cellulose) or longer linker may be necessary for our proposed screen. Lastly, we have generated the necessary reagents (such as RNAi vectors, antibodies) for the second phase of our project. The information obtained from our studies should prove especially useful for the development of new and effective screening strategies, drug targets, and treatment for breast cancer.

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